ProteoSpin

NORGEN BIOTEK CORPORATION

Application Manual

CBED (Concentration, Buffer Exchange and Desalting) MAXI KIT
DETERGENT CLEAN-UP MAXI KIT

For Use with P/Ns 17000 and 17100

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General Introduction

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Norgen's ProteoSpin™ Maxi Kits include the CBED (Concentration, Buffer Exchange and Desalting) Kit and the Detergent Clean-up Kit. The maxi columns contained in these kits are specifically designed for rapid protein purification and isolation using spin-column chromatography technology. The Maxi Kits can process samples containing 0.25 mg to 8 mg of protein. The technology used in these kits is based on Norgen's proprietary chromatography resin, silicon carbide (SiC). The SiC acts as an ion exchanger that is very efficient in concentrating protein solutions, in lowering salts, and in removing greater than 95% of the detergents present.

The ProteoSpin™ CBED Maxi Kit provides a fast and simple procedure for concentrating large volumes of dilute protein solutions for buffer exchange, and for removing different types of salts from protein samples. The kit is highly efficient in removing many different salts commonly used in the laboratory including, but not limited to $MgCl_2$, NaCl, KCl, $CaCl_2$, LiCl, and CsCl. The simultaneous removal of salts while concentrating a dilute protein solution makes the kit a convenient method for preparing proteins before running many downstream applications such as SDS-PAGE and iso-electric focusing. These large columns are frequently used to prepare protein samples for structural analysis where larger amounts of protein are needed, such as X-ray crystallography, NMR spectroscopy and other applications.

Concentrating and desalting protein solutions is a key step in protein sample preparation. Conventional salt removal techniques, including dialysis, spin dialysis, gel filtration, precipitation and solid phase extraction, are time-consuming and, in the case of dialysis, may result in dilution of the protein sample. The use of the Proteospin™ CBED Maxi Kit results in high-quality protein preparations with the proteins concentrated, buffer exchanged, and free of the original sample salts.

The ProteoSpin™ Detergent Clean-Up Maxi Kit provides a fast and simple procedure for removing SDS and other detergents from protein samples. Detergents are extensively used to prepare protein samples; however, detergents must be removed prior to downstream analysis because of their undesirable effects. For example, detergents can often cause poor results in instruments such as mass spectrometers due to large numbers of contaminating peaks and may actually cause physical harm to the instrument. In addition, detergents interfere with purification procedures such as chromatography and gel electrophoresis as well as with structural characterization techniques such as crystallography and amino acid sequence analysis.

Ion Exchange Chromatography

In ion exchange chromatography, ions that are electrostatically bound to an insoluble and chemically inert matrix are reversibly replaced by ions in solution. The matrix can be either an anion exchanger, consisting of positively charged groups that reversibly bind anions in solution, or it can be a cation exchanger bearing negatively charged groups that bind to mobile cations. Proteins are complex molecules that can have an overall positive or negative charge that depends on their amino acid composition and the pH of their solutions. In this case, any given protein will bind to anion or cation exchangers depending on the net charge of the protein. When purifying a protein, the pH and salt concentration of the buffer solution (in which the protein is dissolved) are chosen to immobilize the desired protein on the selected ion exchanger. The impure protein solution is applied to a column that contains the ion exchanger, and has been washed and equilibrated with the buffer solution. The proteins bind to the matrix and are eluted by either changing the salt concentration or the pH.

Silicon Carbide as an Ion Exchanger

The chromatographic resin used in the ProteoSpin™ Maxi Kits consists of silicon carbide (SiC), a man-made material noted for its hardness (second only to diamond) and high resistance to chemical change. These properties make SiC highly suitable for spin column chromatography even at extremes of pH. When processed appropriately, SiC becomes an effective cation exchanger for the purpose of purifying macromolecules. The surface of SiC is negatively charged and can bind positively charged macromolecules. SiC has poor affinity to monovalent and divalent cations, also making it an effective resin for the removal of salts.

Soluble proteins bind to SiC through the interaction between the positively charged side-groups on the polypeptide chain and the negative charges on the SiC surface. As a cation exchanger, SiC has an estimated effective pKa $\approx$ 2 making the resin negatively charged over a wide range of pH. Therefore, with the exception of those peptides that are highly acidic, most soluble polypeptides can be bound to SiC.

Isoelectric Point

Proteins generally contain a number of ionizable groups with a variety of pKa values. For each protein in a solution, there is a particular pH where the negative charges on the molecule exactly balance the positive charges, therefore giving the protein zero net charge. This is known as the isoelectric point, or pI of the protein. The pI of a protein determines the ideal conditions for its purification by ion exchange chromatography. For example, based on pI, one can determine whether the pH of a binding buffer and an elution buffer are suitable for a particular protein. Isoelectric points for a number of proteins have already been determined. If the pI of a protein of interest is unknown, a theoretical pI can be determined using web-based tools found at the Expasy site *http://us.expasy.org/tools/pi_tool.html*. Another useful pI-predictor method can be found at *http://www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html*. It should be noted, however, that the apparent pI of a protein as revealed in isoelectric focusing studies may differ significantly from its theoretical pI.

Removal of Detergents from Biological Samples

Detergents are surface-active agents (surfactants) that contain both a hydrophobic portion and a hydrophilic portion. In aqueous solutions, detergents may form stable structures called micelles with their hydrophilic portions facing the aqueous environment and their hydrophobic portions being hidden in the core.

The charge of the hydrophilic portion enables the classification of detergents into four major groups; anionic, cationic, zwitterionic and non-ionic. Anionic and cationic detergents can be used for processes ranging from solubilizing membrane proteins under non-denaturing conditions to the denaturing conditions of running SDS-PAGE electrophoresis. Common ionic detergents include SDS and sodium deoxycholate. Zwitterionic detergents, such as CHAPS, protect the native state of the protein while preventing protein aggregation during purification procedures. Zwitterionic detergents have low denaturing features whereas non-ionic detergents are non-denaturing. This makes non-ionic detergents suitable for solubilizing membrane proteins without altering biological activity by maintaining the secondary and tertiary structures of the protein. However, these non-ionic detergents, including NP-40, Triton X-100 and Tween 20, are less effective at disrupting protein aggregation. Conventional methods for detergent removal include hydrophobic adsorption, gel chromatography, dialysis, ion-exchange chromatography and precipitation techniques. These methods are time-consuming and tedious and may also result in the loss of protein sample.

The ProteoSpin™ Detergent Clean-Up Maxi Kit is an innovative and user-friendly method that can remove greater than 95% of detergents (based on Triton X-100 and SDS measurements) from protein samples while maintaining high protein recovery. The kit is able to remove all types of detergents including ionic, non-ionic and zwitterionic. It is designed to remove detergents in protein solutions either in their free form or bound form as when complexed with the protein. Detergent molecules that are free in the solution cannot bind to SiC if the detergent is negatively charged because it is repelled by the negative charges on the surface of SiC. Positively charged detergents, however, are expected to preferentially interact with SiC, causing proteins to bind poorly.

By virtue of the polar and nonpolar regions in a detergent molecule, detergents can partition in both hydrophobic and hydrophilic environments. Most detergents unbind from proteins in an aqueous environment in the presence of alcohols, freeing the proteins to interact with water molecules. If the solution is now allowed to move along a chromatographic column containing the SiC resin, the protein molecules bind to the resin while the detergent moves along with the liquid phase. The captured protein can then be eluted into a different buffer free of detergents.

ProteoSpin™ CBED Maxi Kit

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ProteoSpin™ CBED Maxi Kit Benefits

Basic Features	Benefits
Fast and easy processing	Efficient processing of multiple samples in about 30 minutes using an easy-to-use protocol.
No mixing or formulation	Solutions are provided for both acidic and basic proteins with no need for formulation.
No molecular weight cutoff	Based on ion exchange mechanism, a broad size range of proteins (from peptides to large proteins) can be processed.
Proteins bind while salts are discarded in flowthrough	SiC has an inherent low affinity for salt and a high affinity for proteins, hence it provides salt removal and buffer exchange simultaneously.
Suitable for downstream applications	Final elution performed with volatile or non-volatile buffers suitable for: • Mass spectrometry • SDS-PAGE • Isoelectric focusing • X-ray crystallography • NMR spectroscopy
Maxi columns are provided	Allows for binding capacities up to 8 mg.

ProteoSpin™ CBED Maxi Kit Components

- Column Activation and Wash Buffer (Acidic)
- Column Activation and Wash Buffer (Basic)
- pH Binding Buffer (Acidic)
- pH Binding Buffer (Basic)
- Elution Buffer
- Neutralizer
- ProteoSpin™ SiC Maxi Spin Columns (filled with SiC) inserted into 50mL collection tubes
- Final Elution Tubes (50 mL)
- ProteoSpin™ CBED Maxi Kit Application Manual
- ProteoSpin™ CBED Maxi Kit Protocol Card

Recommended Storage Conditions

For unopened solution containers, the reagents should remain stable for 12 months when stored at room temperature. Once opened, all solutions, except for the two binding buffers, should be stored at 4°C when not in use. The binding buffers should remain at room temperature with the lids tightly closed. Salt crystal formation may occur when stored at 4°C. If crystals are visible, bring the entire bottle to room temperature and mix gently to redissolve.

Customer-Supplied Reagents and Equipment

You must have the following in order to use the ProteoSpin™ CBED Maxi Kit:

- Micropipettors
- pH Indicator paper
- Benchtop centrifuge (capable of spinning the 50mL conical tubes)
- Other elution buffers (optional)

Procedure

Overview of CBED Maxi Kit Procedures

The kit utilizes spin columns containing SiC, a cation exchanger that largely depends on pH for its reversible binding capability and is affected minimally by high salt concentrations. Binding occurs if the protein or proteins of interest retain a net positive charge at the binding pH. Non-specifically bound materials such as salts are washed from the column and the bound protein is eluted into a small volume of elution buffer. The process results in an effective concentration and desalting of the protein. Each spin column is able to concentrate and desalt up to 8 mg of acidic or basic protein. Two protocols are described, depending on whether the protein of interest is acidic or basic.

Choosing a ProteoSpin™ Procedure: Acidic or Basic Protocol

The ProteoSpin™ CBED Maxi Kit comes with solutions for concentrating and desalting both acidic and basic proteins. The user must select one or the other protocol that is appropriate to the protein being purified. In theory, the protocol for acidic proteins should apply to a majority of acidic and basic proteins since the resin is a cation exchanger. That is, all proteins with pI greater than the binding pH at 4.5 should bind. Basic proteins, however, bind strongly when they are used under these conditions, making their elution quite inefficient. Therefore, for soluble basic proteins, a different condition for binding the protein to the resin has been developed. For the purposes of this kit, a protein whose pI is less than 8 will be treated as acidic and will use the acidic protocol; otherwise, use the basic protocol. Figure 1 illustrates how to select which procedure to follow for a particular protein given the pI of that protein.

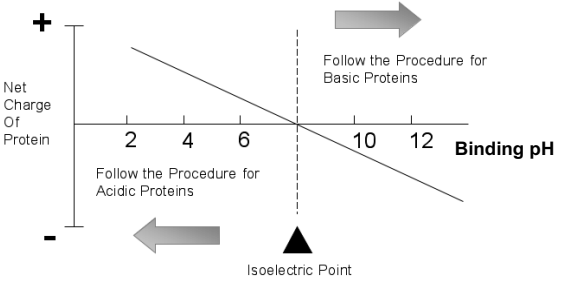

Figure 1. Choosing a procedure based on the isoelectric point (pI)

Protocols

The following procedures describe the concentration and desalting of acidic or basic proteins in either buffered or unbuffered (dissolved in water) solutions.

Note: All centrifugation steps in these procedures are carried out at 1,000 x g in a benchtop centrifuge. Please check your centrifuge's user manual to determine the appropriate speed that gives 1,000 x g. Performance of the kit is not affected by temperature, and thus the procedure may be performed at room temperature, 4ºC, or on ice. The user must take discretionary measures, such as chilling samples in ice, to preserve biological activity.

CBED Protocol for Acidic Proteins

Proteins with isoelectric points (pI) of less than 7 are by definition acidic proteins. However, for purposes of using the kit, the Protocol for Acidic Proteins applies to any protein whose pI is less than 8.0. Proteins with pI higher than 8.0 are purified using the Protocol for Basic Proteins. If the pI of the protein being purified is not known, the theoretical pI may be calculated using the web-based applications at *http://us.expasy.org/tools/pi_tool.html* or *http://www.up.univmrs.fr/~wabim/d_abim/compo-p.html*.

Protocols

A. Sample Preparation

Note: In preparing your sample for column binding by centrifugation, you will mix your protein solution with the pH Binding Buffer (Acidic) provided in the kit to make proper adjustment of the pH. Make sure that all particulates in your sample have been removed by either filtration or centrifugation. The column reservoir has a capacity for 20 mL hence multiple centrifugations will be required for larger volumes.

1. Obtain your protein sample and determine its volume.

2. Depending on your starting protein solution, you will require a certain volume of the pH Binding Buffer (Acidic) to adjust the pH of your protein solution to 4.5. If the starting protein solution is in water, then add one part of the pH Binding Buffer (Acidic) to 49 parts of your protein solution. However, if the starting protein solution already contains a buffer, you may need greater volumes depending on your sample's buffer type and strength. Table 1 (page 16) serves only as guideline for the amount of pH Binding Buffer (Acidic) to use for every milliliter volume of a protein solution in a 100 mM buffer to obtain pH 4.5. Please check the pH after mixing and add more if necessary to obtain the desired pH.

   Please note that if the protein solution is already at the desired pH or lower, do not add any binding buffer.

Table 1. pH Adjustment for Acidic Proteins

Starting pH of Solution	Volume of *pH Binding Buffer (Acidic)* per milliliter of protein solution (based on 100mM buffered solution)
4	0
5	20 µL
6	20 µL
7	20 µL
8	50 µL
9	80 µL
10	80 µL
11	80 µL
12	100 µL

3. Set aside until the protein binding step.

B. Column Activation

1. Obtain a spin column with its 50 mL conical collection tube. Unscrew the cap.

2. Apply 5 mL of Column Activation and Wash Buffer (Acidic) to the column. Screw the cap back on **LOOSELY**.

3. Centrifuge at 1,000 x g for two minutes.

   Note: Start timing spins only after centrifuge has reached desired speed.

4. Repeat steps 2 and 3 to complete the column activation step. Discard the flowthrough.

C. Protein Binding

During the binding step, the protein solution is passed through the resin bed aided by centrifugation. The protein is captured as it comes in contact with the resin. For most protein samples tested, the centrifugation time provides sufficient contact time for the protein to bind.

1. Apply the protein sample (from the Sample Preparation step) onto the column and centrifuge for two minutes. A maximum of 20 mL can be added at one time.

2. Discard the flowthrough. Reassemble the spin column with its collection tube.

   Note: You can save the flowthrough in a fresh tube for assessing your protein's binding efficiency.

3. Depending on your sample volume, repeat steps 1 and 2 until the entire protein sample has been applied to the column.

4. Discard any remaining flowthrough and reassemble the spin column with its collection tube.

D. Column Wash

This step removes non-specifically bound debris from the column.

1. Apply 10 mL of Column Activation and Wash Buffer (Acidic) to the column and centrifuge for two minutes.

2. Discard the flowthrough and reassemble the spin column with its collection tube.

3. Add another 10 mL of Column Activation and Wash Buffer (Acidic) to the column and centrifuge for two minutes.

4. Inspect the column and ensure that the liquid has passed through into the collection tube. There should be no liquid in the column. If necessary, spin an additional two minutes to dry.

E. Protein Elution

The Elution Buffer that is supplied is 50 mM sodium phosphate pH 12.5. Please refer to Appendix 1 (Optional Elution Buffers) for a list of alternate elution solutions that have been tested with the kit. The proteins in the buffer should be neutralized immediately. It is therefore recommended that the Neutralizer that is provided is pre-added to the elution tube (fresh 50 mL conical tube). However, this is not needed for the first elution. For the second elution, 0.3 mL of neutralizer is needed. **Note:** Please verify the pH of the first elution and adjust with Neutralizer if required.

1. Add 300 µL Neutralizer, if desired (not required for 1st elution), to a fresh 50 mL collection tube (supplied).

2. Transfer the spin column, with bound protein, into the 50 mL collection tube from step 1.

3. Apply 4 mL of Elution Buffer to column and centrifuge to elute bound protein.

4. A second elution is optional (another 4 mL). Repeat Steps 1 to 3 if desired. Keep separate from the 1st elution.

Note: Approximately 90% of bound protein is recovered in the first two elutions. If desired, a third elution may be carried out. This should be collected into a different tube (to which Neutralizer is pre-added) to prevent dilution of the first two elutions.

Protein samples are now ready for downstream applications.

CBED Protocol for Basic Proteins

Proteins with isoelectric points (pI) of less than 7 are by definition acidic proteins. However, for purposes of using the kit, the Protocol for Acidic Proteins applies to any protein whose pI is less than 8.0. Proteins with pI higher than 8.0 are purified using the Protocol for Basic Proteins. If the pI of the protein being purified is not known, the theoretical pI may be calculated using the web-based applications at *http://us.expasy.org/tools/pi_tool.html* or *http://www.up.univmrs.fr/~wabim/d_abim/compo-p.html*.

Note: All centrifugation steps in these procedures are carried out at 1,000 x g in a benchtop centrifuge. Please check your centrifuge's user manual to determine the appropriate speed that gives 1,000 x g. Performance of the kit is not affected by temperature, and thus the procedure may be performed at room temperature, 4ºC, or on ice. The user must take discretionary measures, such as chilling samples in ice, to preserve biological activity.

A. Sample Preparation

Note: In preparing your sample for column binding by centrifugation, you will mix your protein solution with the pH Binding Buffer (Basic) provided in the kit to make proper adjustment of the pH. Make sure that all particulates in your sample have been removed by either filtration or centrifugation. The column reservoir has a capacity for 20 mL hence multiple centrifugations will be required for larger volumes.

1. Obtain your protein sample and determine the volume.

2. Depending on your starting protein solution, you will require a certain volume of pH Binding Buffer (Basic) to adjust the pH of your protein solution to 7.0. If the starting protein solution is in water, then add one part of the pH Binding Buffer (Basic) to 49 parts of the protein solution. However, if the starting protein solution is in a buffer, you may need greater volumes depending on the buffer type and strength. Table 2 below serves only as guideline for the amount of pH Binding Buffer (Basic) to use for every milliliter volume of a protein solution in a 100 mM buffer to obtain pH 7. Please check the pH after mixing and add more if necessary to obtain the desired pH.

Table 2. pH Adjustment for Basic Proteins

Starting pH of Solution	Volume of pH Binding Buffer (Basic) per mL of protein solution (based on 100 mM buffered solution)
4	150 µL
5	80 µL
6	80 µL
7	0 µL
8	60 µL
9	60 µL
10	60 µL
11	80 µL
12	80 µL

3. Set aside until the protein binding step.

B. Column Activation

1. Obtain a spin column with its 50 mL conical collection tube. Unscrew the cap.

2. Apply 5 mL of Column Activation and Wash Buffer (Basic) to the column. Screw the cap back on **LOOSELY**.

3. Centrifuge for two minutes at 1,000 x g.

   Note: Start timing only after centrifuge has reached desired speed

4. Repeat steps 2 and 3 to complete the column activation step. Discard the flowthrough.

C. Protein Binding

During the binding step, the protein solution is passed through the resin bed aided by centrifugation. The protein is captured as it comes in contact with the resin. For most protein samples tested, the centrifugation time provides sufficient contact time for the protein to bind.

1. Apply the protein sample (from the Sample Preparation step) onto the column and centrifuge for two minutes. A maximum of 20 mL can be added at one time.

2. Discard the flowthrough. Reassemble the spin column with its collection tube.

   Note: You can save the flowthrough in a fresh tube for assessing your protein's binding efficiency.

3. Depending on your sample volume, repeat steps 1 and 2 until the entire protein sample has been applied to the column.

4. Discard any remaining flowthrough and reassemble the spin column with its collection tube.

D. Column Wash

This step removes non-specifically bound debris from the column.

1. Apply 10 mL of Column Activation and Wash Buffer (Basic) to the column and centrifuge for two minutes.

2. Discard the flowthrough and reassemble the spin column with its collection tube.

3. Add another 10 mL of Column Activation and Wash Buffer (Basic) to the column and centrifuge for two minutes.

4. Inspect the column and ensure that the liquid has passed through into the collection tube. There should be no liquid in the column. If necessary, spin an additional two minutes to dry.

E. Protein Elution

The Elution Buffer that is supplied is 50 mM sodium phosphate pH 12.5. Please refer to Appendix 1 (Optional Elution Buffers) for a list of alternate elution solutions that have been tested with the kit. The proteins in the buffer should be neutralized immediately. It is therefore recommended that the Neutralizer that is provided is pre-added to the elution tube (fresh 50 mL conical tube). For all elutions, 0.3 mL of Neutralizer is needed

1. Add 300 µL Neutralizer to a fresh 50 mL collection tube (supplied).

2. Transfer the spin column, with bound protein, into the 50 mL collection tube from step 1.

3. Apply 4 mL of Elution Buffer to column and centrifuge to elute bound protein.

4. A second elution is optional (another 4 mL). Repeat Steps 1 to 3 if desired. Keep separate from the 1st elution.

   Note: Approximately 90% of bound protein is recovered in the first two elutions. If desired, a third elution using Elution Buffer may be carried out. This should be collected into a different tube (to which Neutralizer is pre-added) to prevent dilution of the first two elutions.

Protein samples are now ready for downstream applications.

ProteoSpin™ Detergent Clean-Up Maxi Kit

ProteoSpin™ Detergent Clean-Up Maxi Kit Benefits

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Basic Features	Benefits
Fast and easy processing	In contrast to longer dialysis procedures, the easy-to-use protocol allows processing of a number of samples in about 40 minutes.
A complete kit for acidic and basic protein samples	The kit contains essential solutions for processing detergent-containing samples from 0.25 - 8 mg of protein. Solutions are provided for processing both acidic and basic proteins.
No detergent carryover	Unlike HPLC procedures, each sample is processed individually using its own spin tube providing no sample carryover or column bleed from sample to sample.
Usable with a variety of detergents	This kit can be used to remove detergents including, but not limited to, SDS, Triton X-100, CHAPS, NP-40 and Tween 20.
Efficient detergent removal	Removes more than 95% of detergents for many proteins, allowing tryptic digestion without residual detergents that interfere with trypsin digestion.
Maxi columns are provided	Allows for binding capacities up to 8 mg.

ProteoSpin™ Detergent Clean-Up Maxi Kit Components

- Column Activation and Wash Buffer (Acidic)
- Column Activation and Wash Buffer (Basic)
- pH Binding Buffer (Acidic)
- pH Binding Buffer (Basic)
- Elution Buffer
- Neutralizer
- ProteoSpin™ SiC Maxi Spin Columns (filled with SiC) inserted into 50 mL collection tubes
- Final Elution Tubes (50 mL)
- ProteoSpin™ Detergent Clean-Up Maxi Kit Application Manual
- ProteoSpin™ Detergent Clean-Up Maxi Kit Protocol Card

Recommended Storage Conditions

For unopened solution containers, the reagents should remain stable for 12 months when stored at room temperature. Once opened, all solutions, except for the two binding buffers, should be stored at 4°C when not in use. The binding buffers should remain at room temperature with the lids tightly closed. Salt crystal formation may occur when stored at 4°C. If crystals are visible, bring the entire bottle to room temperature and mix gently to redissolve.

Customer-Supplied Reagents and Equipment

You must have the following in order to use the ProteoSpin™ Detergent Clean-Up Maxi Kit:

- Micropipettors
- pH Indicator paper
- Other Elution Buffers (optional)
- Benchtop Centrifuge (capable of spinning the 50mL tubes)
- Isopropanol
- Milli Q water
- 100 mL sterile bottles

Procedure

The ProteoSpin™ Detergent Clean-Up Maxi Kit is designed to remove detergents in solutions of acidic or basic proteins. Detergents are removed by phase partitioning with cation exchange chromatography and the general principle for removal applies to different classes of detergents.

The process applies to a wide variety of soluble proteins ranging from acidic to basic proteins. Two procedures, one for acidic proteins and another for basic proteins, are described. In theory, the protocol for acidic proteins should apply to a majority of acidic and basic proteins since the resin is a cation exchanger; that is, all proteins with a pI greater than the binding pH at 4.5 should bind reversibly. However, basic proteins bind strongly when they are used under these conditions, making their elution quite inefficient. Therefore, for soluble basic proteins (pI ≥ 8), a different condition for binding the protein to the resin has been developed. For the purposes of this kit, a protein whose pI is less than 8 will be treated as acidic and will use the acidic protocol; otherwise, use the basic protocol.

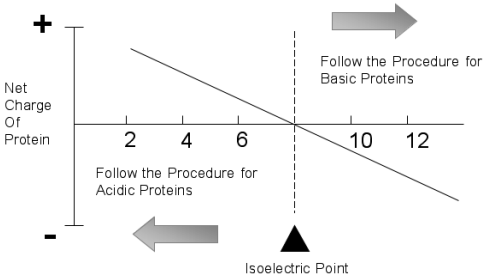

Figure 2. Choosing a procedure based on the isoelectric point (pI)

Protocols

The following procedures describe the removal of detergents from acidic or basic proteins in either buffered or unbuffered (dissolved in water) solutions. The kit utilizes spin columns containing SiC, which bind the protein of interest if it retains a net positive charge. Non-specifically bound proteins and detergents are washed from the column and the specific protein is eluted into specially formulated elution buffer, removing the detergent from the protein sample. Each spin column will remove detergent from up to 8 mg of acidic or basic protein. This kit is designed to remove a variety of detergents and/or surfactants from protein solutions. It is capable of removing detergents such as Triton X-100, SDS, Chaps, NP-40 and Tween 20. Each column has a capacity of up to 8 mg of protein, and will remove greater than 95% of detergents when present.

Detergent Clean-Up Protocol for Acidic Proteins

Proteins with isoelectric points (pI) of less than 7 are by definition acidic proteins. However, for purposes of using the kit, the Protocol for Acidic Proteins applies to any protein whose pI is less than 8.0. Proteins with pI higher than 8.0 are purified using the Protocol for Basic Proteins. If the pI of the protein being purified is not known, the theoretical pI may be calculated using the web-based applications at *http://us.expasy.org/tools/pi_tool.html* or *http://www.up.univmrs.fr/~wabim/d_abim/compo-p.html*.

Note: All centrifugation steps in these procedures are carried out at 1,000 x g in a benchtop centrifuge. Please check your centrifuge's user manual to determine the appropriate speed that gives 1,000 x g. Performance of the kit is not affected by temperature, and thus the procedure may be performed at room temperature, 4ºC, or on ice. The user must take discretionary measures, such as chilling samples in ice, to preserve biological activity.

A. Sample Preparation

Note: In preparing your sample for column binding by centrifugation to removed detergents, you will mix your protein solution with the pH Binding Buffer (Acidic) provided in the kit to make proper adjustment of the pH. Isopropanol (not supplied with the kit) will also be added to aid in the process. Make sure that all particulates in your sample have been removed by either filtration or centrifugation. The column reservoir has a capacity for 20 mL hence multiple centrifugations will be required for larger volumes.

1. Obtain your protein sample and determine the volume.

2. Depending on your starting protein solution, you will require a certain volume of pH Binding Buffer (Acidic) to adjust the pH of your protein solution to 4.5. If the starting protein solution is in water, then add one part of the pH Binding Buffer (Acidic) to 49 parts of the protein solution. However, if the starting protein solution is in a buffer, you will need greater volumes of the pH Binding Buffer (Acidic) depending on the buffer type and strength. Table 3 below serves only as guideline for the amount of pH Binding Buffer (Acidic) to use for every milliliter volume of a protein solution in a 100 mM buffer to obtain pH 4.5. Please check the pH after mixing and add more if necessary to obtain the desired pH.

Please note that if the pH is at 4.5 or lower, do not add any binding buffer.

Table 3. pH Adjustment for Acidic Proteins

Starting pH of Solution	Volume of pH Binding Buffer (Acidic) per mL of protein solution (based on 100 mM buffered solution)
4	0 µL
5	20 µL
6	20 µL
7	20 µL
8	50 µL
9	80 µL
10	80 µL
11	80 µL
12	100 µL

3. Add one volume of isopropanol to the pH-adjusted solution.

4. Check the pH after mixing and add more pH Binding Buffer (Acidic) if necessary to obtain the desired pH.

5. Set aside until the protein binding step.

B. Preparation of Modified Column Activation and Wash Buffer (Acidic)

This step prepares a modified solution to activate and wash the columns. It is prepared using the provided pH Binding Buffer (Acidic) and isopropanol.

1. Combine the following in a sterile 100 mL bottle and mix well:

   - 2 mL pH Binding Buffer (Acidic)
   - 50 mL isopropanol
   - 48 mL sterile deionized water

2. Label the container as Modified Column Activation and Wash Buffer (Acidic) and keep it tightly sealed at room temperature when not in use. This volume is sufficient for the entire Detergent Clean-up Maxi Kit.

C. Column Activation

1. Obtain a spin column with its 50 mL conical collection tube. Remove lid.

2. Apply 5 mL of Modified Column Activation and Wash Buffer (Acidic) to the column. Screw the cap back on **LOOSELY**.

3. Centrifuge for two minutes at 1,000 x g.

   Note: Start timing only after centrifuge has reached desired speed.

4. Repeat steps 2 and 3 to complete the column activation step. Discard flowthrough.

D. Protein Binding

During the binding step, the protein solution is passed through the resin bed aided by centrifugation. The protein is captured as it comes in contact with the resin. For most protein samples tested, the centrifugation time provides sufficient contact time for the protein to bind.

1. Apply the protein sample (from the Sample Preparation step) onto the column and centrifuge for two minutes. A maximum of 20 mL can be added at one time.

2. Discard the flowthrough. Reassemble the spin column with its collection tube.

   Note: You can save the flowthrough in a fresh tube for assessing your protein's binding efficiency.

3. Depending on your sample volume, repeat steps 1 and 2 until the entire protein sample has been applied to the column.

4. Discard any remaining flowthrough and reassemble the spin column with its collection tube.

E. Column Wash

This step removes non-specifically bound debris from the column.

1. Apply 10 mL of Modified Column Activation and Wash Buffer (Acidic) to the column and centrifuge for two minutes.

2. Discard the flowthrough and reassemble the spin column with its collection tube.

3. Add 10 mL of **Regular** Column Activation and Wash Buffer (Acidic) to the column and centrifuge for two minutes.

4. Inspect the column and ensure that the liquid has passed through into the collection tube. There should be no liquid in the column. If necessary, spin an additional two minutes to dry.

F. Protein Elution

The Elution Buffer that is supplied is 50 mM sodium phosphate pH 12.5. Please refer to Appendix 1 (Optional Elution Buffers) for a list of alternate elution solutions that have been tested with the kit. The proteins in the buffer should be neutralized immediately. It is therefore recommended that the Neutralizer that is provided is pre-added to the elution tube (fresh 50 mL conical tube). However, this is not needed for the first elution. For the second elution, 0.3 mL of Neutralizer is needed. **Note:** Please verify the pH of your first eluted protein sample and adjust with the Neutralizer if required.

1. Add 300 µL Neutralizer, if desired (not required for the 1st elution), to fresh 50 mL collection tube.

2. Transfer the spin column, with bound protein, into the 50 mL collection tube from step 1.

3. Apply 4 mL of Elution Buffer to column and centrifuge to elute bound protein.

4. A second elution is optional (another 4 mL). Repeat Steps 1 to 3 if desired. Keep separate from the 1st elution.

Note: Approximately 90% of bound protein is recovered in the first two elutions. If desired, a third elution using Elution Buffer may be carried out. This should be collected into a different tube (to which the Neutralizer is pre-added) to prevent dilution of the first two elutions.

Protein samples are now ready for downstream applications.

Detergent Clean-Up Protocol for Basic Proteins

Proteins with isoelectric points (pI) of less than 7 are by definition acidic proteins. However, for purposes of using the kit, the Protocol for Acidic Proteins applies to any protein whose pI is less than 8.0. Proteins with pI higher than 8.0 are purified using the Protocol for Basic Proteins. If the pI of the protein being purified is not known, the theoretical pI may be calculated using the web-based applications at *http://us.expasy.org/tools/pi_tool.html or http://www.up.univmrs.fr/~wabim/d_abim/compo-p.html*.

Note: All centrifugation steps in these procedures are carried out at 1,000 x g in a benchtop centrifuge. Please check your centrifuge's user manual to determine the appropriate speed that gives 1,000 x g. Performance of the kit is not affected by temperature, and thus the procedure may be performed at room temperature, 4ºC, or on ice. The user must take discretionary measures, such as chilling samples in ice, to preserve biological activity.

A. Sample Preparation

Note: In preparing your sample for column binding by centrifugation to remove detergents, you will mix your protein solution with the pH Binding Buffer (Basic) provided in the kit to make proper adjustment of the pH. Isopropanol (not supplied with the kit) will also be added to aid in the process. Make sure that all particulates in your sample have been removed by either filtration or centrifugation. The column reservoir has a capacity for 20 mL hence multiple centrifugations will be required for larger volumes.

1. Obtain your protein sample and determine its volume.

2. Depending on your starting protein solution, you will require a certain volume of the pH Binding Buffer (Basic) to adjust the pH of your protein solution to 7.0. For example, if the starting protein solution is in water, add one (1) part of the pH Binding Buffer (Basic) to 49 parts of the protein solution. If the starting protein solution is in a buffer, you may need greater volumes of the pH Binding Buffer (Basic) to lower the pH, depending on the buffer type and its ionic strength. Table 4 below serves only as guideline for the amount of pH Binding Buffer (Basic) to use for every milliliter volume of a protein solution in a 100 mM buffer to obtain pH 7.0. Please check the pH after mixing and add more if necessary to obtain the desired pH.

Table 4. pH Adjustment for Basic Proteins

Starting pH of Solution	Volume of pH Binding Buffer (Basic) per mL of protein solution (based on 100 mM buffered solution)
4	150 µL
5	80 µL
6	80 µL
7	0 µL
8	60 µL
9	60 µL
10	60 µL
11	80 µL
12	80 µL

3. Add one volume of isopropanol to the pH-adjusted solution.

4. Mix contents well and measure the pH before applying to the column. Further adjust the pH if necessary.

5. Set aside until the protein binding step.

B. Preparation of Modified Column Activation and Wash Buffer (Basic)

This step prepares a modified solution to activate and wash the columns. It is prepared using the provided pH Binding Buffer (Basic) and isopropanol.

1. Combine the following in a sterile 100 mL bottle and mix well:

   - 2 mL of pH Binding Buffer (Basic)
   - 50 mL of isopropanol
   - 48 mL of sterile deionized water

2. Label the container as Modified Column Activation and Wash Buffer (Basic) and keep it tightly sealed at room temperature when not in use. This volume is sufficient for the entire Detergent Clean-Up Maxi Kit.

C. Column Activation

1. Obtain a spin column with its 50 mL conical collection tube. Unscrew the cap.

2. Apply 5 mL of Modified Column Activation and Wash Buffer (Basic) to the column. Screw the cap back on **LOOSELY**.

3. Centrifuge for two minutes at 1,000 x g.

Note: Start timing only after centrifuge has reached desired speed.

4. Repeat steps 2 and 3 to complete the column activation step. Discard the flowthrough.

D. Protein Binding

During the binding step, the protein solution is passed through the resin bed aided by centrifugation. The protein is captured as it comes in contact with the resin. For most protein samples tested, the centrifugation time provides sufficient contact time for the protein to bind.

1. Apply the protein sample (from the Sample Preparation step) onto the column and centrifuge for two minutes. A maximum of 20 mL can be added at one time.

2. Discard the flowthrough. Reassemble the spin column with its collection tube.

   Note: You can save the flowthrough in a fresh tube for assessing your protein's binding efficiency.

3. Depending on your sample volume, repeat steps 1 and 2 until the entire protein sample has been applied to the column.

4. Discard any remaining flowthrough and reassemble the spin column with its collection tube.

E. Column Wash

This step removes non-specifically bound debris from the column.

1. Apply 10 mL of Modified Column Activation and Wash Buffer (Basic) to the column and centrifuge for two minutes.

2. Discard the flowthrough and reassemble the spin column with its collection tube.

3. Add 10 mL of **Regular** Column Activation and Wash Buffer (Basic) to the column and centrifuge for two minutes.

4. Inspect the column and ensure that the liquid has passed through into the collection tube. There should be no liquid in the column. If necessary, spin an additional two minutes to dry.

F. Protein Elution

The Elution Buffer that is supplied is 50 mM sodium phosphate pH 12.5. It is recommended that upon elution, the protein solution is neutralized immediately especially for proteins that are known to be sensitive to high pH. The following steps are suggested if the supplied Elution Buffer is used and neutralization is desired. For a list of alternate elution solutions that have been tested for the kit, please refer to Appendix 1 (Optional Elution Buffers).

1. Add 300 µL of Neutralizer to a fresh 50 mL collection tube (supplied)

2. Transfer the spin column, with bound protein, into the 50 mL collection tube from step 1.

3. Apply 4 mL of Elution Buffer to column and centrifuge to elute bound protein.

4. A second elution is optional (another 4 mL). Repeat steps 1 to 3 if desired. Keep separate from the 1st elution.

   Note: Approximately 90% of bound protein is recovered in the first two elutions. If desired, a third elution using Elution Buffer may be carried out. This should be collected into a different tube (to which Neutralizer is pre-added) to prevent dilution of the first two elutions.

   Protein samples are now ready for downstream applications.

Appendix 1.
Optional Elution Buffers

A

Proteins bound to SiC via interactions with electrostatic charges are eluted through pH-dependent mechanisms. The efficiency of protein elution depends on high pH above the pI of the protein to be purified. The pH of the elution buffer chosen must be at least one unit higher than the pI (isoelectric point) of the protein of interest. Solutions not provided with the ProteoSpin™ Maxi CBED or Detergent Clean-Up Maxi kits may be utilized if they are more appropriate for your needs. The table below describes optional elution buffers and their observed efficiency when BSA is used as a test protein.

Table 5. Alternate Elution Buffers

Elution Buffers	Approximate Protein Recovery
50 mM ammonium hydroxide (approximate pH 11)	70%
250 mM ammonium hydroxide (approximate pH 11)	70%
1 M ammonium hydroxide (approximate pH 11)	90%
1 M ethanolamine (approximate pH 9)	70-80%
50 mM sodium phosphate (approximate pH 12.5)	95%
500 mM sodium phosphate (approximate pH 12.5)	<70%
100 mM sodium borate (approximate pH 12.5)	95-100%
1 M Tris (approximate pH 12.5)	95%

Appendix 2.
Proteins with Established Isoelectric Points

B

Table 6. Examples of Proteins with Known Isoelectric Points

Protein	Molecular Weight (kDa)	Iso-Electric Point (pI)
Albumin, bovine serum	67	5.5
Albumin, human serum	66.5	4.8
Carbonic anhydrase	30	7.3
Carboxypeptidase	34	6.0
Catalase	250	5.6
Cytochrome C	13	10.6
Fibrinogen	330	5.5
Growth hormone, human	21.5	6.9
Hemoglobin, horse	65	6.9
Immunoglobulin G	150	6.4-7.2
Insulin	5.7	5.3
Lysozyme, hen egg white	14.3	11.0
Myoglobin, horse	17	7.0
Ovalbumin	40	4.6
Pepsin	35.5	< 1.0
Ribonuclease	14	7.8
Thyroglobulin	660	4.6
Trypsin inhibitor, soybean	22.5	4.55
Urease	480	5.1

Appendix 3.
Frequently Asked Questions and Troubleshooting

C

Question	CBED	Detergent Removal	Answer
What is the dark gray material that is packed into the spin columns?	•	•	The dark gray chromatography material is silicon carbide (SiC). It is processed using proprietary non-chemical methods to function as an ion exchange chromatography resin.
How does the kit remove detergents?		•	The kit removes detergents from aqueous solutions by taking advantage of the poor affinity of detergents to silicon carbide, the chromatographic resin used. The resin works in conjunction with isopropanol to eliminate detergents including those that bind to proteins such as SDS. Please refer to the application note "Efficient Removal of SDS from Proteins Solutions" on our website at http.//www.norgenbiotek.com.
How does the kit concentrate and desalt proteins?	•		The resin has high affinity for proteins with net positive charges, but has very poor affinity for monovalent and divalent cations. When dilute protein solutions with high salt pass through the resin, the charged proteins are captured by the resin while the aqueous solution with the salts is removed as the flowthrough. The bound protein can be recovered by eluting it in small volumes and in salt-free buffers to achieve several-fold concentration.

Question	CBED	Detergent Removal	Answer
What proteins can be processed using this kit?	•	•	All soluble (acidic and basic) proteins can be processed using this kit.
What can I do if I do not know the isoelectric point (pI) of my protein of interest?	•	•	The isoelectric point of a protein (pl) may be theoretically computed from its amino acid composition through the aid of web-based applications such as http://us.expasy.org/tools/pi_tool.html. Another site at http://www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html determines isoelectric points (pl) with considerations for position of residues in the polypeptide chain. The calculated pl should help determine whether the acidic or basic protocol is to be followed. Alternatively, if there is sufficient protein sample, the protein can be bound and eluted using first the acidic protocol, and then the basic protocol, and the recovery compared to determine which protocol gives better results.
Can the columns be re-used?	•	•	Columns are designed for single use only. If used more than once, sample carry-over may cause cross-contamination.
How should I store the kit solutions?	•		Once opened, the solutions in their containers should be stored at 4 °C when not in use, except the pH Binding Buffers, which remains at room temperature with the lids tightly closed. If any precipitation of the reagents occurs, warm to room temperature and dissolve the crystals prior to use. If unopened, the solution containers may be stored at room temperature.

Question	CBED	Detergent Removal	Answer
Do I need to pre-filter my sample before loading it onto the column?	•	•	Insoluble materials, if present in the protein solution, should be spun down and the clarified solution applied to the column.
How should I prepare my sample before loading it onto the column?	•	•	Ensure that the pH of the sample is the same as the pH of the Column Activation and Wash Solution that you are using. Also, ensure that the amount of protein in your sample will not overload the column capacity (8 mg).
What are the maximum and minimum sample volumes that I can load onto the column?	•	•	We recommend that no more than 20 mL of sample be loaded onto the column, to ensure that the tip of the column does not rest in the flowthrough that is caught in the collection tube. Excess volumes can be loaded in subsequent spins as long as the collection tube is emptied between loads. The minimum load should be at least 4 mL to completely cover the resin bed.
What are the maximum and minimum amounts of protein that I can load onto the column?	•	•	We recommend between 250 μg and 8 mg of sample be loaded onto the column in order to obtain high percentage recovery.
What are the highest and lowest recommended microcentrifuge speeds for the columns?	•	•	We recommend a speed around 1,000 x g in the benchtop centrifuge. Speeds of more than 1,500 x g should be avoided. Lower speeds can be investigated to maximize efficiency. Additional spinning times may be required to ensure all the liquid has passes through.

Question	CBED	Detergent Removal	Answer
What is the typical amount of protein recovered that I can expect with the ProteoSpin™ columns?	•	•	Recovery of BSA in water was found to be greater than 90% when testing 8 mg of protein. Recovery depends on the specific protein sample, and varies with detergent concentrations. If detergents are present, use the Detergent Clean-Up Kit.
Can I autoclave the columns?	•	•	No. The columns are not designed to be autoclaved as it causes the plastic in the columns to deform.
Can all types of detergents be removed using this kit? What about SDS?		•	Yes, the kit is capable of removing all types of detergents including anionic, cationic, zwitterionic and nonionic. SDS can be removed efficiently with the kit, including tightly bound SDS complexed with proteins.
Do any detergents or reagents interfere with the performance of the columns?	•		Most commonly used biological detergents have little or no effect on column performance except for SDS. Also, greater than 1M concentration of urea and guanidine salts are known to interfere with protein binding. For the removal of detergents, use the ProteoSpin Detergent Clean-Up Kit.
How can I tell if my protein bound correctly to the column?	•	•	Save the flowthrough from the binding step and perform a protein assay to determine the amount of protein. To determine the amount of protein present, use a reliable colorimetric assay. Several are commercially available. To eliminate inaccuracies, make sure that the protein standard used is in the same buffer condition as the flowthrough.

Question	CBED	Detergent Removal	Answer
I used the recommended binding pH, but still my protein did not bind correctly. Can I use a different binding pH?	•	•	Yes. The binding pH may be adjusted depending on the pI of your protein. It is suggested to start with a pH that is one unit lower than the pI, and if necessary, lowering the pH from there. If the provided pH binding buffer is used, it can be adjusted to a pH as low as 3.7 by titrating it with HCl or glacial acetic acid. For proteins with an even lower pI, the pH binding buffer may be substituted with a 500 mM phosphate buffer (prepared by mixing 9 parts of 500 mM phosphoric acid with 1 part 500 mM trisodium phosphate) and used as a 10-fold concentrate.
Am I able to check the activity of my protein by using the eluted protein directly?	•	•	If the elution buffer provided in the kit was used, then the eluted protein may be used directly to check activity of the protein. The elution buffer is sodium phosphate and is compatible with many assays for biological activity.
I use a colorimetric protein assay to determine concentration of eluted proteins. Is there an accurate way of doing it?	•	•	The colorimetric protein assays available in the market are quite accurate, however, there are a number of ways to improve them. First, using a linear regression to fit the points for the standard curve is often less accurate than using a second-degree polynomial. Microsoft Excel™ has full features for curve fitting. The goodness-of-fit for the regression should determine whether first-degree (linear) or second-degree (polynomial) would be used. Second, the protein used as the standard should closely match the dye-binding characteristics of the protein of interest. For example, using BSA as a standard to determine concentrations of immunoglobulins may provide erroneous results. This is because immunoglobulins do not bind some dyes efficiently. Third, ensure that the protein standard is in a solution that resembles the solution of the test protein. Sometimes buffer constituents can have drastic effects on dye binding properties.

Question	CBED	Detergent Removal	Answer
The acidic and basic methods are almost identical, except for the solutions used. Why is it necessary to separate the two?	•	•	In ion exchange chromatography, it is customary to use either a cation or anion exchanger resin, depending on the type of target protein. For SiC resin, this carries negative charges on its surface, proteins bind through positive charges on their side chains that are exposed on the surface. In theory, the acidic method should suffice to capture all basic and acidic proteins. In practice, however, we find that basic proteins bound under acidic conditions are difficult to elute, and therefore decrease the overall performance of the columns. Binding basic proteins at pH 7 reduces column affinity for such proteins, making elution more efficient.
The binding pH is 4.5, yet my protein has a pI of 4. Do I need to adjust the pH of my binding solution to lower than 4.5? If so, how would I do that?	•	•	If your protein has a pI lower than 4.5, it is almost always necessary to lower the pH of the protein solution so that it is one pH unit lower than the protein's pI. If the ProteoSpin™ pH Binding Buffer for Acidic Proteins is used, the final pH may be adjusted to as low as 3.75 by titrating with 100 mM HCl. If a lower binding pH is desired, you may use different buffers, such as phosphate ($pKa_1 = 2.15$). Many acidic proteins tend to precipitate as the pH of the solution approaches their pI. If protein precipitation is apparent (solution becomes cloudy), diluting the solution to a lower protein concentration may solve the problem. Using a binding pH of one-half unit below the protein's pI is also feasible, although the expected recovery for the bound protein will be lower.

Question	CBED	Detergent Removal	Answer
When desalting my basic protein, my protein yield is generally poor. Why is this and what is the best way to overcome the problem?	•		Basic proteins generally are more difficult to recover from ion exchange columns than acidic proteins. The problem usually lies in basic proteins' high affinity to negatively charged ion exchange resins. Elution of these proteins may require a combination of high pH and high ionic strength (by adding salt) in the elution buffer. Although SiC has low affinity for salts, which allows it to be an effective desalter, high salts can affect proteins severely. For basic proteins, the effect is reduction of binding efficiency. To overcome the problem, dilute the basic protein solution so that the salt concentration is 0.2 M or lower, then use the kit to concentrate the protein and desalt at the same time. Note that recovery of the basic protein increases with decreasing salt concentration achieved by dilution. Fortunately, the ability of acidic proteins to bind does not seem to be affected by high salt concentrations, hence the normal protocol may be used without modification.
How can I separate an acidic protein from a basic one when both are present in the same solution?	•	•	Separation of proteins using SiC ion exchange chromatography is achieved by differential binding of proteins due to their pI. When separating an acidic protein from a mixture with a basic protein, use the Basic Protocol.

Question	CBED	Detergent Removal	Answer
Can I purify a protein in a glycerol-containing solution?	•		Glycerol of less than 20% concentration has little effect on the binding of most proteins to the column. At higher glycerol concentrations (as high as 60% v/v) , the recovery rate may be reduced by as much as 50%. Note that the ProteoSpin™ Detergent Clean-Up Kit must not be used with proteins that contain glycerol.
Can I purify a protein in a sugar-containing solution?	•	•	Sucrose has very little effect, even at as high a concentration as 1M.

Appendix 4.
Troubleshooting Guide

D

Each of the following tables discusses a problem which can occur, its possible causes, and presents solutions and explanations.

Problem: Protein Solution Does Not Flow Through the Column

Possible Causes	Solution and Explanation
Centrifugation speed was too low.	Check the centrifuge and ensure it is capable of generating 1,000 x g. Sufficient centrifugal force is required to push the liquid through the column.
Inadequate spin time	Spin an additional minute or two to ensure the liquid is through the column.
Protein solution is too viscous	Dilute the protein solution and adjust the pH to either pH 4.5 or 7. Highly viscous materials due to high protein concentrations can slow down flow rate significantly.
Cellular debris is present in the protein solution	Filter the sample with a 0.45µM filter or spin down insoluble materials and transfer liquid portion to the column. Solid, insoluble materials can cause severe clogging problems.
Protein solution is not completely dissolved	Dissolve the sample in a larger amount of buffer. Solid, insoluble materials can cause clogging problems.

Problem: Poor Protein Recovery

Possible Causes	Solution and Explanation
Incorrect procedure was used.	Ensure that the acidic protocol was used for acidic proteins and the basic protocol was used for basic proteins. It is known that when basic proteins are bound with the acidic protocol, elution is inefficient because the basic proteins are bound too tightly.
Initial volume of sample applied to the column was too low.	Load at least 4 mL onto the column. This volume ensures that the entire bed is covered sufficiently.
Incorrect pH adjustment of samples.	Ensure that the pH of the sample is 4.5 for acidic proteins and pH 7.0 for basic proteins.
Protein may have precipitated prior to loading onto the column.	If the pH of the protein solution is the same as the pI of the protein, precipitation may occur. In this case, adjust the pH of the sample to at least one pH lower than the pI of your protein.

Problem: Eluted Protein is Degraded

Possible Causes	Solution and Explanation
Eluted protein was not neutralized.	Add 300 µL of Neutralizer to each 4mL of eluted protein for all basic protein elutions. Acidic proteins tend not to need the neutralization for the first elution but require it for the second or third. Check the pH of the first elution if you are using an acidic protein.
Eluted protein solution was not neutralized quickly enough.	If eluted protein is not neutralized immediately, degradation will occur. We strongly recommend adding Neutralizer in order to lower the pH.
Proteases may be present.	Use protease inhibitors during all steps of the Sample Preparation.
Bacterial contamination of the protein solution.	Prepare the protein sample with 0.015% sodium azide. The elution buffer already contains sodium azide.